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A study on organo-sulfur tolerant bacterial strains from extremophilic coal mine environment- isolation and growth factors

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Present study describes the isolation of three organo-sulfur tolerant microbial strains designated NA-1, NA-2 and OF-SD (isolated at pH 7) from samples of Assam (India) coal mines. NA-1, NA-2 and OF-SD were tested positive for utilization of ~100mM thiophene and ~50 mM each of phenyl disulphide and dibenzothiophene in presence of media with extremely good growth pattern (generation time). Of the three strains, OF-SD characterized as *Bacillus thioparans* by >1200bp 16S ribosomal RNA partial gene sequencing was having an excellent generation time of 0.6h. These bacterial isolates are now tested for their ability to degrade the sulfur inclusions in coal, which will be further beneficial for industrial aspects.

[**Keywords**: Coal mines; pyrite; organic sulfur; bacteria; desulfurization]

Introduction

In coal, sulfur present in the form of inorganic and organic sulfur. Inorganic sulfur in the form of sulphide such as pyritic sulfur (FeS_2), sphalerite (ZnS), Galena (PbS), etc. and sulphates such as Barite ($BaSO_4$), Gypsum ($CaSO_4.2H_2O$) [1,2]. On the other hand organic sulfur are present in the form of: aliphatic or aromatic thiols (marcaptane, thiophenol), aliphatic, aromatics or mixed sulphides (thioethers) and heterocyclic compound (dibenzothiopene) etc [1,2]. During the processing of coal combustion the sulfur is oxidized and forms SO_2 which causes acid deposition which is detrimental to the soil, agriculture and ecosystem[3].So before the utilization of the coal it should be desulfurized to avoid these above stated phenomena. Desulfurization comprises of physical, chemical and biological process. Here we focus on the biodesulfurization, a process where microbes are used to desulfurize the coal.

Many microorganisms were isolated and they were able to remove the pyritic sulfur and organic sulfur. Removal pyritic sulfur is found effective, but removal of organic sulfur is quite not easy. But some of the bacteria are capable to degrade the organic sulfur to utilize the sulfur as their sole source of sulfur. *Aspergillus* sp. isolated from Assam coal could eliminate 78% of total sulfur from Assam coal[4]. Thermophilic microorganism *Sulfolobus acidocaldorius* can remove 96% inorganic sulfur and about 50% of total sulfur from coal[5]. A microorganism metabolically related to *Xanthomonas maltophila*, was able to remove both organic and inorganic sulphur at neutral pH, with efficiencies of 69% and 68% respectively[6]. Degradation of dibenzothiophene (DBT) occurs by cleavage of C-C bond, C-S bond present in the structure of dibenzothiopene. C-S bond cleavage performed by *Rhodococcus erythropolis*, *Rhodococcus rhodochrous* IGTS8 at 30°C, *Paenibacillus sp.* A11-2, *Bacillus* k10 etc., have been reported [7-10]. Coal is suitable environment of diverse kind of microorganisms so the bacteria present in coal are able to degrade the organic sulfur compound present in the coal for their nutrition. To desulfurize the organic compounds present in the coal, bacteria contain specific genes (dsz) that codes for specific enzymes[11].

In this study, bacterial strains which could utilize organosulfur compounds were isolated from coal samples and oil contaminated soil sample collected. Strains with good tolerance to organosulfur compounds like DBT, phenyl disulphide (PDS), thiopene were selected and identified. The ability of the selected strains for the removal of organic sulfur from high sulfur coal was also investigated.

Materials and Methods

The sulfur-removing bacteria in this study were isolated from Tirap Mines of NECL, Assam, India. Dibenzothiopene (DBT), Phenyl disulphide (PDS), Thiopene were purchased from Sigma-Aldrich. Nutrient broth, agar agar was obtained from Hi-media, Folin-Ciocalteau from Sisco research laboratories Pvt. Ltd. All chemicals were of analytical grade and commercially available.

For isolation of sulfur utilizing bacteria, the coal sample was serial diluted and streaked over nutrient agar slant (NA-1). Coal core sample was extracted by boiling with water and serial diluted. This extracted coal sample was streaked over the nutrient agar slant (NA-2). Deeply-dug soil sample near abandoned coal dumps was serially diluted and streaked over nutrient agar slant for isolation of OF-SD. Slants are kept in incubator at 35ºC, pH-7.0. After 24 hrs of incubation growth was observed. Biochemical characterization and carbohydrate fermentation test were performed. Growth effect on sulfur was monitored by enriching the medium with different organo-sulfur compounds such as DBT, PDS and Thiopene. The nutrient broth medium with sulfur contained (g/L): Nutrient Broth-13, DBT/PDS/Thiopene (varied concentration), pH-7.0. Grown culture of NA-1, NA-2, OF/SD were inoculated in various organosulfur rich nutrient broth and kept for incubation at 35^{o}C for 7 days. All the isolates have been tested positive for utilization of organosulfur compounds up to 100mM thiopene, 50 mM of Phenyl disulphide and 10 mM of Dibenzothiopene concentration in presence of media. The adapted strains are currently being tested for their potential ability to remove organic sulfur from the coal. To determine the amount of protein produced by NA-1, NA-2 and OF/SD isolates, estimation of protein was done by Lowry's method.

Results and Discussion

Three strains were isolated from the Assam coal mines sample. They were capable of degrading the organic sulfur. NA-1 isolate is single, long rods, yellowish in color, gram negative in nature and having smooth surface (Fig.1a). NA-2 isolate is arranged in chain, long rods, whitish in color, and gram positive with rough colony surface (Fig.1b). OF/SD isolate is arranged in chain, long rods, whitish in color, and gram positive with smooth colony surface (Fig.1c). Morphological and physiological characters of three isolates are shown in Table 1. The NA-1 isolate shows indole negative, methyl red negative, vogus- proskauer negative, citrate utilization positive; i.e., able to utilize citrate as sole source of carbon, catalase negative, triple iron sugar positive. The NA-2 isolate gives indole negative, methyl red positive, vogus-proskauer negative, citrate utilization negative; i.e., unable to utilize citrate, catalase negative, triple iron sugar positive results.

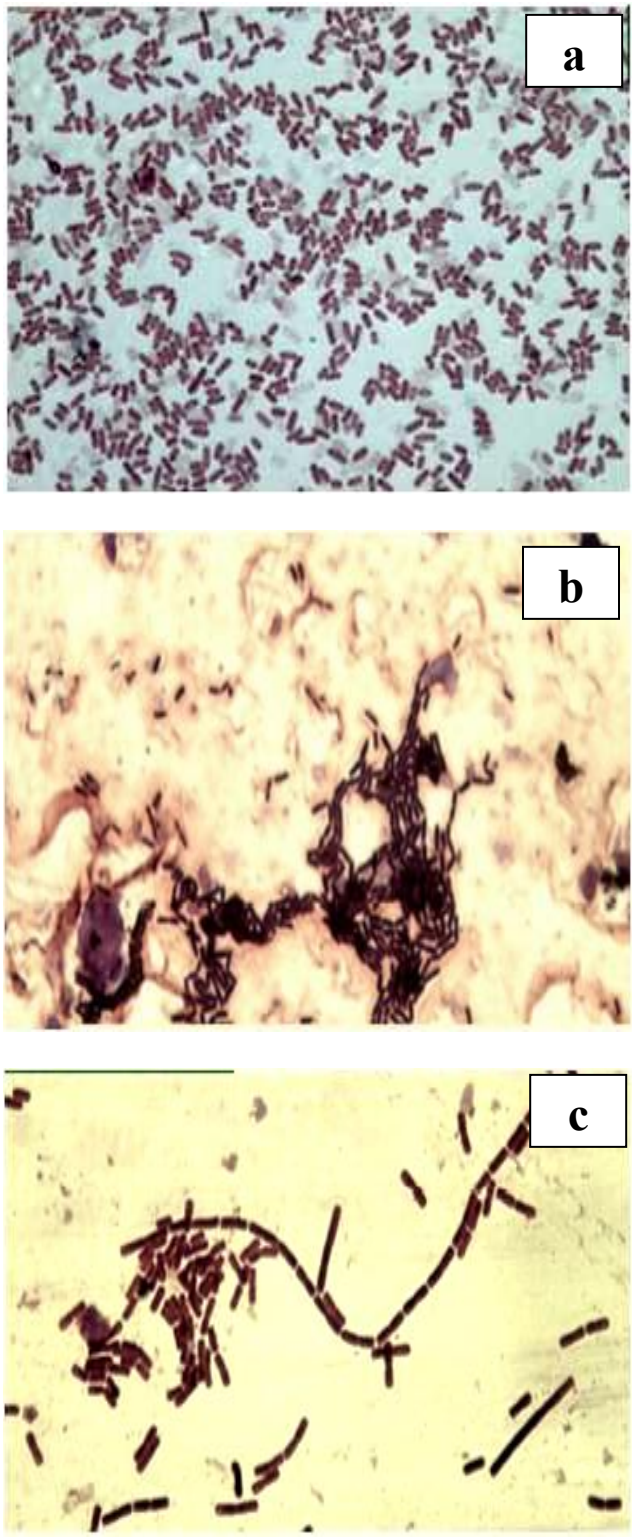


Fig.1- Pure culture of isolates (a) NA-1, (b) NA-2, (c) OF-SD

OF/SD isolate showed indole negative, methyl red positive, vogus-proskauer negative, citrate utilization negative, catalase negative, triple sugar iron test positive. Ability of carbohydrate metabolism of these isolates was tested by using a

comprehensive carbohydrate test kit (Hi-Media). The test is based on the principle of pH change and substrate utilization. On incubation, the organisms undergo metabolic changes which are indicated by spontaneous color change.

Table 1- Morphological and physiological characteristics of the isolates

MORPHOLOGICAL CHARACTER	NA-1	NA-2	OF-SD
Gram's staining	Negative	Positive	Positive
Shape	Short rod	Long rod	Long rod
Arrangement	Single	Bunches	Chains
Color	Yellow	Whitish	Whitish
Colony Surface	Smooth	Rough	Smooth

NA-1 isolate showed positive result for different type of carbohydrate like galactose, L-Arabinose, Rhamnose (monosaccharide's), lactose (disaccharides) and negative result for fructose, xylose (monosaccharide's),sucrose ,cellobiose (di-saccharides) utilization. NA-2 isolate showed positive result for fructose, galactose, xylose, Rhamnose (monosaccharide's), lactose, sucrose (di-saccharides) and negative result for L-Arabinose, cellobiose utilization. OF/SD isolate showed positive result for monosaccharide's like fructose, galactose, xylose, Rhamnose, L-Arabinose utilization and di-saccharides like lactose ,sucrose and cellobiose utilization. Biochemical characterization of the isolates is given below in Table 2.

Table 2- Biochemical characterization of the three isolates derived from coal mine samples

TEST	NA-1	NA-2	OF-SD
Indole test	Negative	Negative	Negative
Methyl–Red test	Negative	Positive	Positive
Vogus-Proskauer	Negative	Negative	Negative
Citrate utilization	Positive	Negative	Negative
Catalase test	Negative	Negative	Negative
Triple Iron sugar	Positive	Positive	Positive
Monosaccharide's			
(A) Fructose	Negative	Positive	Positive
(B) Xylose	Negative	Positive	Positive
(C) Galactose	Positive	Positive	Positive
(D) L-Arabinose	Positive	Negative	Positive
(E) Rhamnose	Positive	Positive	Positive
Di-saccharides			
(A) Lactose	Positive	Positive	Positive
(B) Sucrose	Negative	Positive	Positive
(C) cellobiose	Negative	Negative	Positive

The growth pattern of the three strains was investigated by culturing the in nutrient media. Measurement of turbidity using a spectrophotometer and determination of the cell count using a Petroff Hauser chamber were carried out at an interval of 1 h to study their growth kinetics with measurement of optical density. ln (Cell count (t) / initial cell count) vs. time, also called a first order plot was made and a straight line was fit in to obtain specific growth rate (μ). μ was calculated from the slope of the straight line from which generation time was deduced[12].

In case of NA-1 and NA-2, the generation time at pH 7 and 37^{o}C was estimated to be 2 h; whereas for strain OF-SD, the generation time was calculated to be 0.6 h. Protein estimation was carried out as per Lowry's method[13]. These results shows that the amount of protein increased per hour as their optical density increased after 8 hours of incubation. After initial inoculation (0 h) of bacterial culture NA-1, the optical density (OD) of the culture medium was 0.0407 and the concentration of protein extracted at that time was 0.11mg/mL. As per standard procedure, consecutive 8 hr samples were collected and the concentration of protein reached up to 0.49mg/mL when optical density of the culture was noted to be 1.7020 at 540 nm as shown in Fig.2a. In case of the NA-2 bacterial culture, just after zero hours, the optical density at initial time was 0.0135 with protein concentration equaling 0.14mg/mL. Growth after 8h resulted in OD to rise to 0.5906 and protein concentration to escalate to 0.37 mg/ml (Fig.2b). In case of OF-SD, the optical density of the culture medium was 0.0653 and the concentration of protein was 0.17mg/mL at initial time zero. After 8 hours, the concentration of protein reached 0.89mg/mL with corresponding OD being 1.9323 at 540 nm as seen in Fig.2c.

All of the three isolates are adapted in various concentrations of dibenzothiopene, phenyl disulphide and thiopene. These isolates can utilize 100mM thiopene (Fig.3), 50 mM of phenyl disulfide (Fig.4) and 10mM of dibenzothiopene concentration (Fig.5) in presence of media. The adaptive tolerance of the isolates at various concentrations is shown in Table 3.

From Fig.5 and Table 3, it could be ascertained that OF-SD strain isolated from coal samples, were prolific in growth, and showed high degree of adaptive tolerance of all the proven organ-sulfur compounds prevalent in extremophilic coal environment.

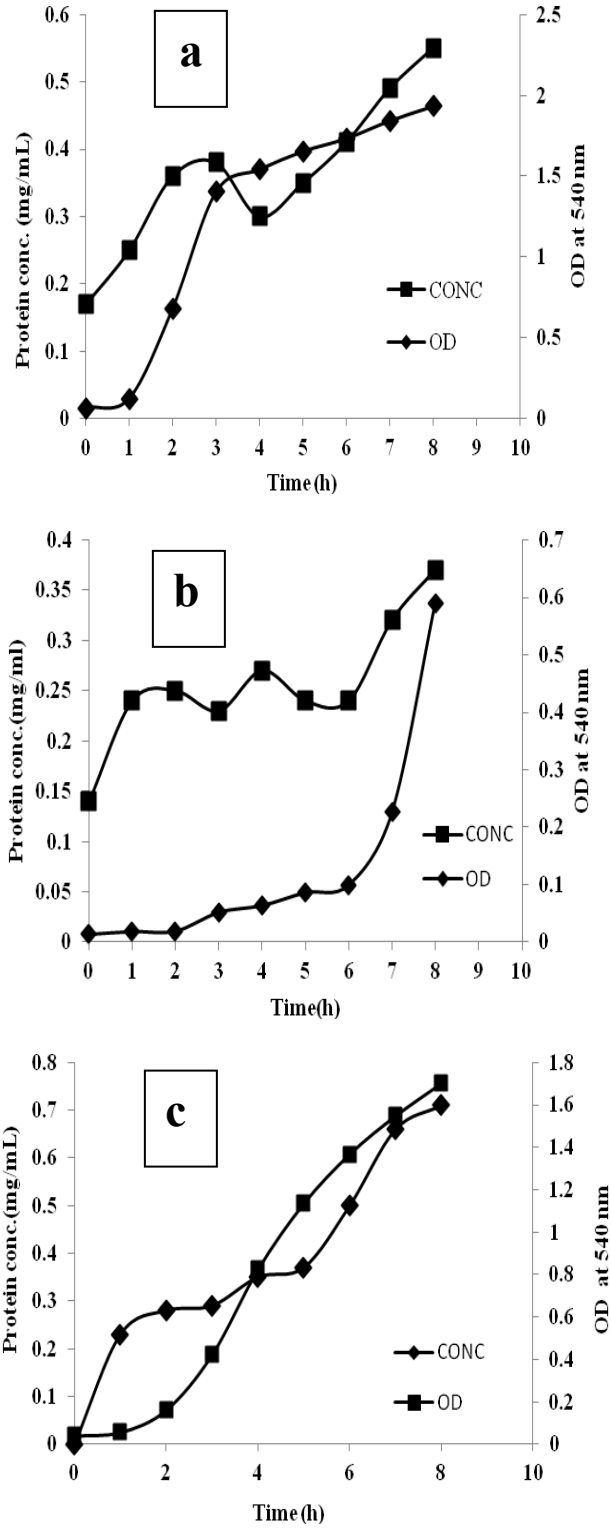


Fig.2- Variation of protein concentration in respect to their optical density of (a) NA-1, (b) NA-2, (c) OF-SD.

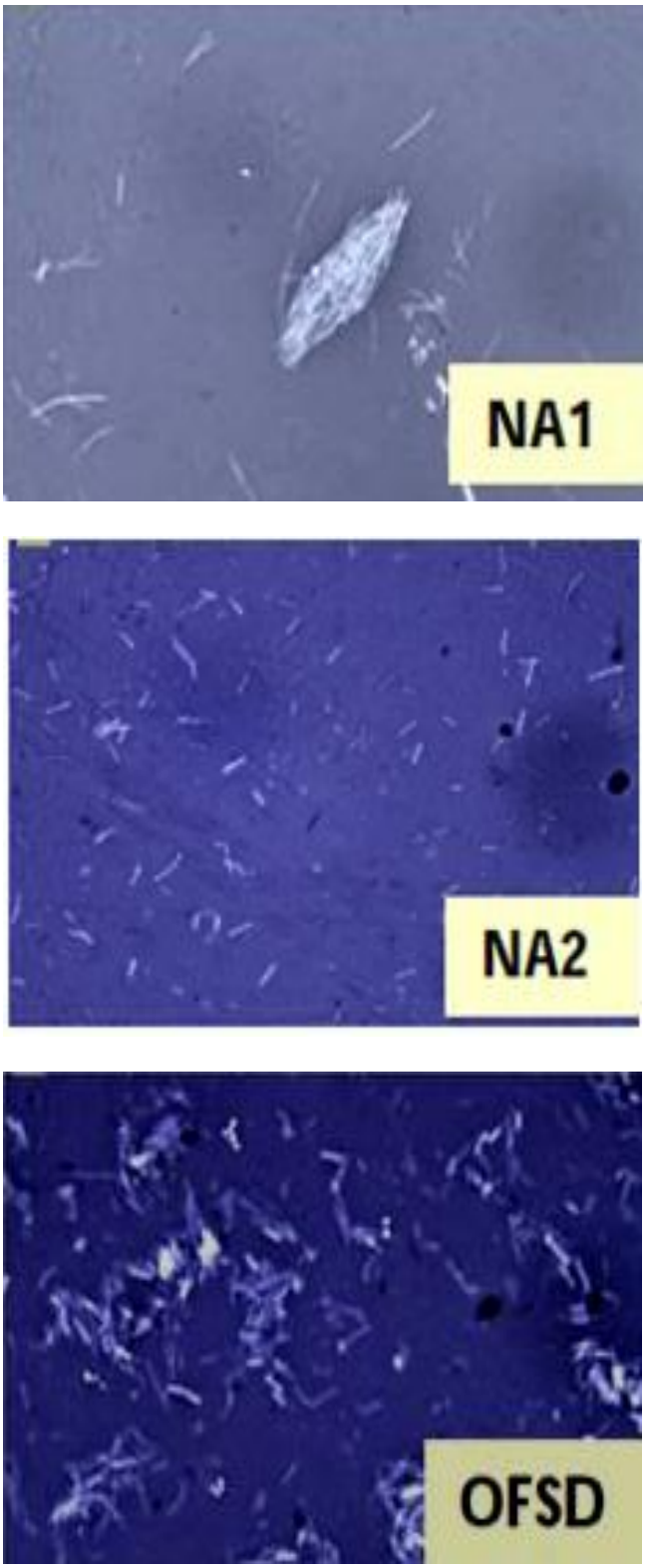
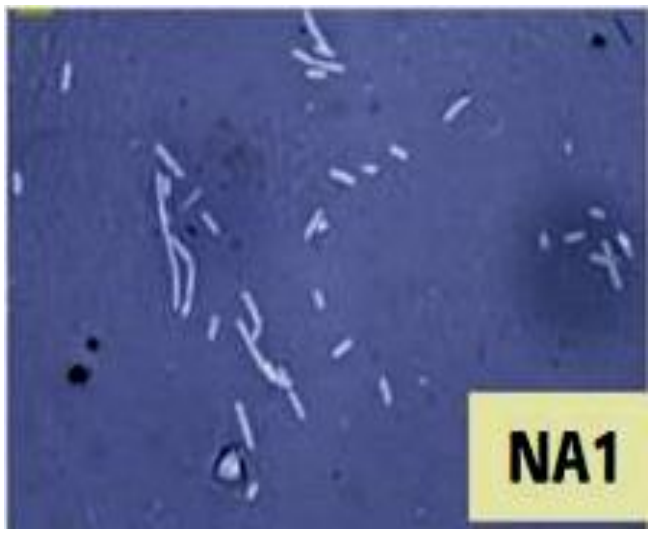
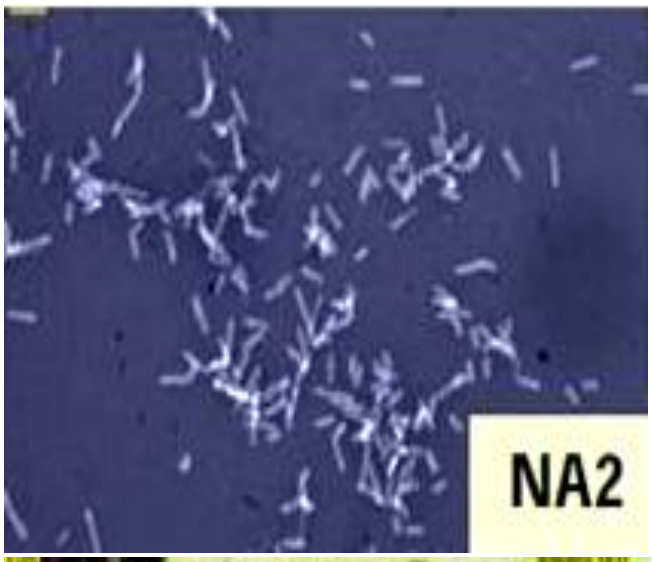


Fig.3- Isolates adapted in 10mM of dibenzothiophene





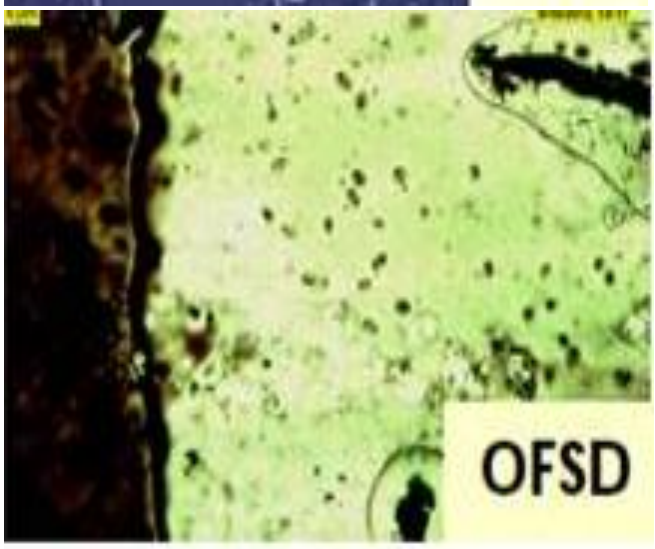
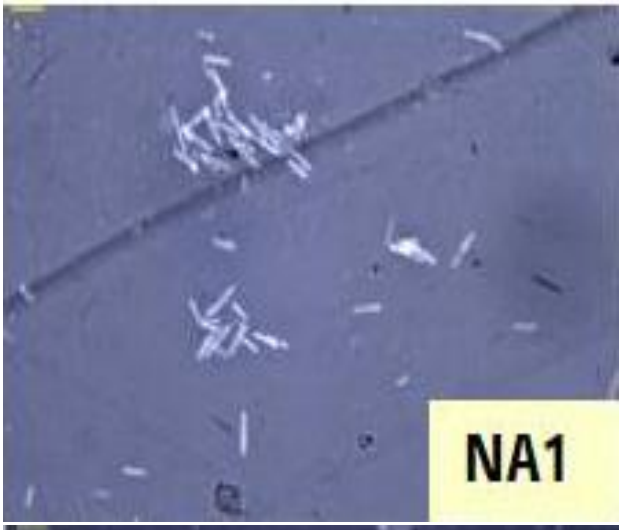
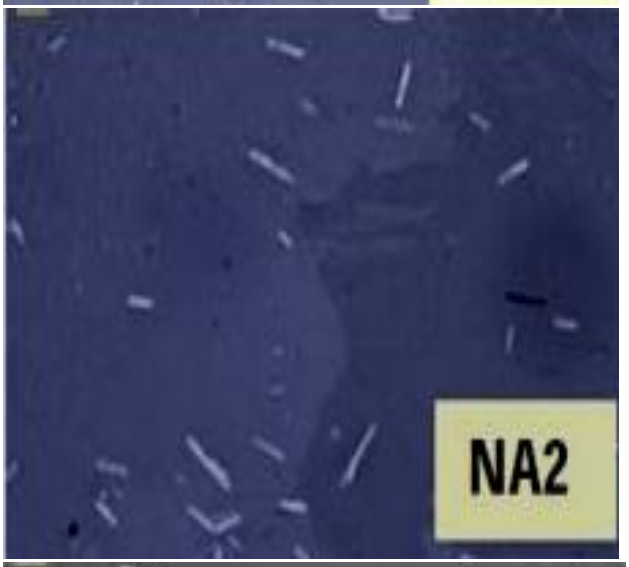


Fig.4- Isolates adapted in 50mM of phenyl disulphide.





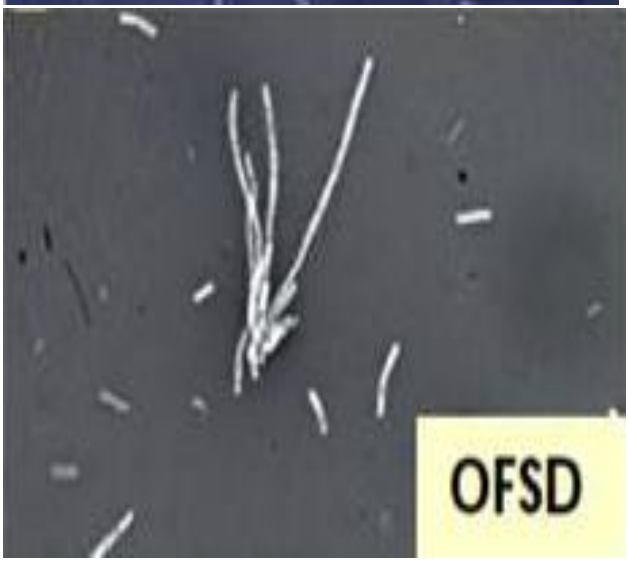


Fig.5- Isolates adapted in 100 mM of thiopene.

Table 3- Growth of the bacteria on various organo-sulfur substrates (A-DBT, B-PDS, C-Thiophene)

Substrate concentration	NA-1			NA-2			OF-SD		
	A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
1mM	+	+	+	+	+	+	+	+	+
2mM	+	+	+	+	+	+	+	+	+
5mM	+	+	+	+	+	+	+	+	+
10mM	+	+	+	+	+	+	+	+	+
100mM	-	+	+	-	+	+	-	+	+

+ indicates growth of bacteria observed under microscope.
- indicates no growth of bacteria observed under microscope.

The purified isolates were subjected to molecular characterization based on 16S rRNA and the results were matched with RDP Database and the confidence in identification was evaluated by both the availability and the extent of homology shown by the ~1200 bp sequence of your sample with its closest neighbor in the database. Pure slant of OF-SD was submitted to Microbial Culture Collection of National Center for Cell Sciences, Pune, INDIA for molecular characterization and classification. It was evaluated and confirmed with 100% homology to *Bacillus thioparans*.

Conclusions

The following major conclusions can be drawn based on the above studies. Three distinct extremophilic bacterial species were isolated from the sulfur rich coal of Tirap Mines, Assam, were found to grow well at neutral pH. Species were able to utilize comprehensively all monosaccharide and disaccharides, indicating the importance of carbon in coal for their growth, and their chemolithitrophic nature. In case of NA-1 and NA-2, the generation time at pH 7 and 37^{o}C was estimated to be 2 h; whereas for strain OF-SD, the generation time was calculated to be 0.6 h. NA-1, NA-2 and OF-SD exhibited a maximal protein concentration of 0.49, 0.37 and 0.89mg/mL in 8h. OF-SD species possessed a relatively higher growth rate. Adaptive tolerance was also established for various organ-sulfur compounds (DBT, Thiophene, PDS) wherein the LD_{100} was 100mM thiopene, 50 mM phenyl disulfide and 10mM dibenzothiophene in presence of media. Molecular characterization of OF-SD reveals its 100% homology to *Bacillus thioparans*, which would further be applied for coal bio-desulphurization studies.

Acknowledgements

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